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# Preservation of protein in wilted lucerne using formic, sulphuric or trichloroacetic acid†

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## Abstract

A laboratory-scale experiment was conducted with lucerne (*Medicago sativa*) to determine the effects of acid treatment on proteolysis during ensiling and during subsequent *in vitro* ruminal protein incubations. Lucerne [300 g dry matter (DM) $kg^{-1}$ forage] was either untreated (control) or treated with sulphuric, formic or trichloroacetic acid (a protein precipitant that stops enzyme activity) at levels sufficient to adjust immediately forage pH to 4·0, then conserved as either silage or hay. Time-course data indicated that non-protein nitrogen (N) formation was 70–90% complete after 1 d of fermentation in the silo. Non-protein N concentrations (g $kg^{-1}$ total N) were 177 at ensiling and increased to 567 (control), 426 (sulphuric), 398 (formic) and 263 (trichloroacetic) after 60 d of ensiling. Because non-protein N in silage treated with formic and sulphuric acids was nearly three times greater than that in silage treated with trichloroacetic acid, it is clear that the typical acid treatments only slow proteolysis and do not destroy protease activity during ensiling. The ruminal protein degradation rate of conserved forages was slower than that of fresh-cut forage that was preserved with dry ice immediately after cutting. The degradation rate of all acid-treated forages was similar, indicating a consistent effect on ruminal degradation regardless of method of preservation. There was a clear effect of acid treatment on reducing the rate and extent of ruminal degradation of protein in lucerne hay.

## Introduction

Conservation of forages as silage results in extensive breakdown of the native plant proteins to non-protein N (NPN), which consists principally of free amino acid nitrogen (AA N), peptide-bound AA N and ammonia nitrogen ($NH_3$ N) (Oshima and McDonald, 1978). The NPN often accounts for more than half of the total N in lucerne (*Medicago sativa*) silage (Muck, 1987) and results in inefficient N utilization by ruminant livestock (Broderick, 1995). Treatments that effectively reduce NPN formation during ensiling include heat (Charmley and Veira, 1990; Carpintero and Suarez, 1992), γ-irradiation (Heron *et al.*, 1986; Charmley and Veira, 1991), and the application of aldehydes (Kung *et al.*, 1986) or formic acid (Barry *et al.*, 1970; Nagel and Broderick, 1992). The last treatment is of particular interest because formic acid has been commonly used as a preservative for direct-cut silage in northern Europe and has dramatically improved the efficiency of N utilization for milk production when applied to wilted [380 g dry matter (DM) $kg^{-1}$ forage] lucerne silage (Nagel and Broderick, 1992). However, it is not known whether formic acid has any effect on proteolysis of the plant proteins beyond acidification.

The objectives of this study were to evaluate the effects of formic acid on silage NPN formation and subsequent ruminal protein degradation, relative to sulphuric acid, merely an acidifying agent, and trichloroacetic acid (TCA), an organic acid that precipitates proteins and inhibits enzyme activity (White *et al.*, 1968).

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## Materials and methods

### *Forage harvest and ensiling*

Fourth-cutting lucerne was cut at prebloom maturity using a mower-conditioner. It was field wilted for 48 h (to approximately 300 g DM $kg^{-1}$ forage) and then chopped with a forage harvester (theoretical length of cut of 1·0 cm). Approximately 20 kg was transported to the laboratory. A sample (Fresh) was also obtained immediately after mowing and held on dry ice for subsequent lyophilization to evaluate the ruminal protein degradability of the native plant proteins. Upon arrival at the laboratory, 1200-g batches of forage were weighed into large plastic tubs and the appropriate amount of acid to adjust forage pH to 4 (determined previously by titration of lucerne macerates) was applied with a misting sprayer, and the forages were hand mixed. This pH was chosen because it approximated to the lowest pH obtainable in lucerne silage made by natural fermentation. Acids were added at rates (mequiv. acid $kg^{-1}$ forage) of 147 (sulphuric), 215 (formic) and 167 (TCA). The ensiling procedure (Muck, 1987) consisted of placing 50 g of forage into 100-ml polypropylene centrifuge tubes with intermittent packing with a wooden dowel. Tubes were capped with butyl rubber stoppers fitted with gas traps, then incubated in a 30 °C water bath. After silos were filled, 400 g of each treatment was placed in a greenhouse for 72 h (final DM content > 800 g $kg^{-1}$ forage) to simulate drying for haymaking. These samples were included in ruminal protein degradation studies to determine the effect of the acids on ruminal protein degradation, independent of the ensiling process.

Triplicate silos from each treatment were opened after 1, 2, 3, 4, 7 and 60 d of ensiling. Silage DM content was determined on samples from each silo by drying for 60 °C for 48 h. A 15·0-g sample from each silo was placed in a blender jar, diluted with distilled water to 150 g and macerated for 30 s in a high-speed blender, then filtered through two layers of cheesecloth. The filtrate was immediately analysed for pH. An aliquot of 10 ml of 25% (w/v) TCA was added to 40 ml of the filtrate to precipitate protein. The solution was then centrifuged at 25 000 × ***g*** for 25 min and the supernatant was analysed for $NH_3$ and free amino acids (AAs) (Broderick and Kang, 1980). Non-protein N was measured by the Kjeldahl method and an autoanalyser (AOAC, 1980) using 10 ml of the TCA supernatant. Peptide-bound AAs were determined by the increase in free AA in the TCA supernatant after digestion with 6 N HCl for 21 h at 105 °C under an $N_2$ atmosphere. The remaining material from each silo, as well as all hays, were then lyophilized and ground in a hammer mill to pass a 1-mm screen. These samples were then analysed for DM (105 °C for 24 h), total N (AOAC, 1980) and ruminal protein degradability.

### *Ruminal protein degradation*

Samples of hays and 60-d silages were assayed for the rate of ruminal protein degradation ($k_d$), the potentially degradable fraction (fraction B) and ruminal protein escape (ruminal undegraded protein) using inhibitor *in vitro* methodology (Broderick, 1987). According to this procedure, protein substrates are incubated with mixed ruminal microbes in the presence of inhibitors that prevent the uptake of free AAs and $NH_3$ (chloramphenicol and hydrazine sulphate). Thus, subtracting the N present in these end products of protein degradation from the amount of substrate protein N initially present allows for computation of the fraction of protein degraded. Analyses were conducted in triplicate using a limited substrate (LS) methodology (Broderick, 1987) as well as by non-linear regression analysis of the integrated Michaelis–Menten (IMM) equation (Broderick and Clayton, 1992). A standard lucerne hay from our laboratory, plus this standard hay treated with an amount of TCA equivalent to that used in silage preparation but added to the incubation vessel immediately before the addition of ruminal inoculum (standard hay + TCA), were included with the LS and IMM incubations. This was done to assess the effects of acid treatment on ruminal protein degradation resulting from acids acting directly on the microbial population rather than on the forage proteins.

### *Statistical analyses*

Data for silage composition are presented graphically, with s.e.m. bars included at each treatment × time point mean ($n = 3$) to indicate variation. Data on composition and *in vitro* ruminal protein degradability of day 60 silages were analysed by analysis of variance (ANOVA) using the general linear models procedure of SAS (1985). Effects included in the model were treatment for silage composition data or treatment and replicate for ruminal protein degradation characteristics. When the $F$ test indicated a significant ($P < 0{\cdot}05$) treatment effect, mean separations were conducted using least significant difference. Estimates of ruminal degrad-

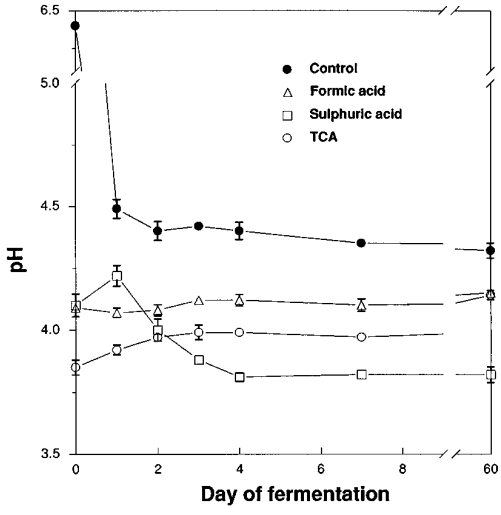


**Figure 1** Changes in pH of lucerne silage over time. Error bars indicate the standard error of treatment × time means ($n = 3$).

ability obtained with the standard hay and standard hay + TCA were compared separately using a *t*-test because these samples represented a different forage source than that used in all other treatments.

## Results and discussion

### *Ensiling*

Initial DM concentrations of silages ranged from 288 to 300 g kg$^{-1}$ forage, very near the target of 300. Initial forage pH was 6·40, declining to 4·49 and 4·32 after, respectively, 1 and 60 d of fermentation in the control silage (Figure 1), indicating a rapid and stable fermentation. Moreover, the profile of N fractions in the control silage (Table 1) was typical of that observed in field-scale lucerne silage (Broderick *et al.*, 1990), indicating that the laboratory ensiling procedure approximated farm-scale ensiling.

Acid application immediately reduced forage pH to 3·85–4·09 (Figure 1), near the intended pH of 4·0. The pH of all acid-treated silages remained below the control silage throughout the ensiling period. Changes in pH with time occurred in sulphuric acid- and TCA-treated silages but these acids, with p*K* values of 1·81 and 1·92 (sulphuric) and 0·70 (TCA), have little buffering capacity at pH 4. In contrast, pH changes with time were negligible in formic acid silage. Formic acid, with a p*K* of 3·75, would have more effectively buffered any fermentation products.

As expected, proteolysis was extensive in the untreated silage; NPN accounted for over half of the total N (Table 1). All acids reduced NPN formation during ensiling; however, the effect of TCA was much greater than that of either sulphuric or formic acids, which were similar to one another. McKersie (1985) determined that free AA release was approximately 31% greater from lucerne ensiled at an initial pH of 4·5 vs. lucerne ensiled at pH 4·0. Moreover, McKersie (1985) found that the activity of partially purified lucerne proteases was lower at pH 4·0 than at pH 4·5. Therefore, some of the differences in NPN content of acid-treated vs. control silage in our experiment were probably due to the differences in pH. Time-course data (Figure 2a) indicated that proteolysis proceeded actively for 3–7 d, except in TCA-treated silage, where there was little NPN formation beyond 1 d. These findings were consistent with those of Muck (1987) in that the largest changes in all NPN fractions occurred within the first 24 h after ensiling. Thus, it is critical that prospective treatments aimed at reducing proteolysis be effective immediately upon ensiling. Silage treated with TCA differed from all other silages in that peptide-bound AA N, rather than free

**Table 1** pH and proportions of total N in NPN fractions in day 60 lucerne silages†

| | | Proportion of total N (g kg$^{-1}$ total N) in | | | |
|---|---|---|---|---|---|
| Treatment | pH | Total NPN‡ | Ammonia N | Free AA N | PBAA N |
| Control | 4·32$^{a}$ | 567$^{a}$ | 74·2$^{a}$ | 342$^{a}$ | 198$^{a}$ |
| Sulphuric acid | 3·82$^{c}$ | 426$^{b}$ | 24·2$^{b}$ | 187$^{b}$ | 183$^{a}$ |
| Formic acid | 4·15$^{d}$ | 398$^{b}$ | 17·7$^{c}$ | 194$^{b}$ | 203$^{a}$ |
| TCA | 4·17$^{b}$ | 263$^{c}$ | 6·7$^{d}$ | 72$^{c}$ | 128$^{b}$ |
| s.e.m. | 0·02 | 9 | 1·0 | 4 | 11 |

Means within columns not sharing a common letter differ ($P < 0·05$).
†NPN, non-protein N; AA N, amino acid nitrogen; PBAA N, peptide-bound AA N; TCA, trichloroacetic acid.
‡Mean NPN content of all forages at ensiling was 177 g kg$^{-1}$ total N.

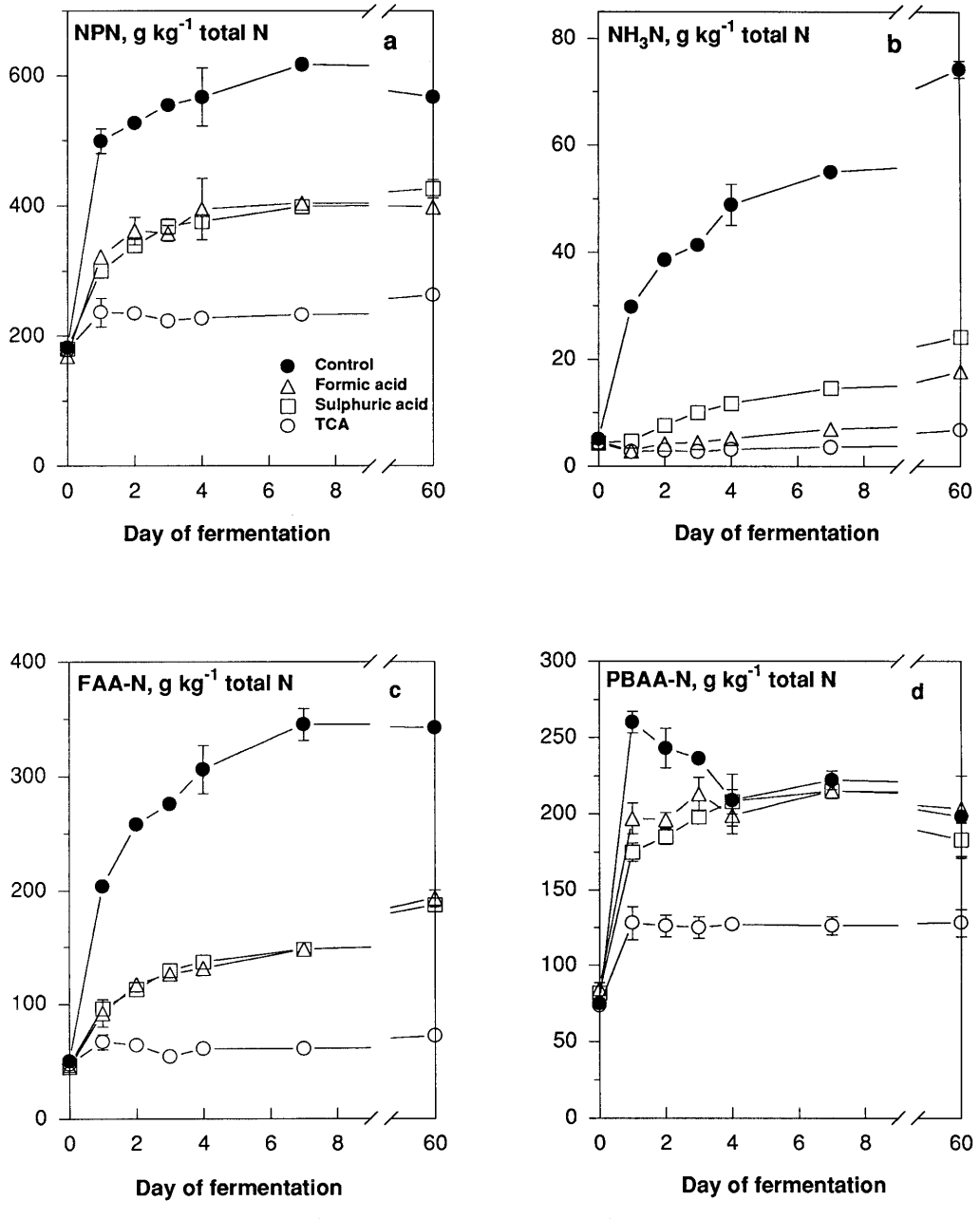


**Figure 2** Changes in lucerne silage content of (a) non-protein N (NPN) (b) $NH_3$ N (c) free (amino acid) AA N and (d) peptide-bound AA N (PBAA N) over time. Error bars indicate the standard error of treatment × time means ($n = 3$).

AA N, accounted for the largest fraction of NPN. Ammonia N content of the NPN decreased the most with acid treatment: $NH_3$ N content was reduced to 33% (sulphuric acid), 24% (formic acid) and about 10% (TCA) of that in control silage. The similar effects on $NH_3$ concentration in silages treated with sulphuric and formic acids was consistent with the findings of O'Kiely *et al.* (1989). Because $NH_3$ N is largely a product of microbial, rather than plant, enzyme activity (Oshima and McDonald, 1978), the acid treatments appeared to restrict microbial as well as plant metabolism. The superiority of TCA

vs. sulphuric and formic acids in reducing proteolysis probably reflected direct inhibition of plant proteases by TCA by virtue of its known properties as a protein precipitant and denaturant (White *et al.*, 1968). However, the safety of treating silage with TCA has not been established and TCA is not approved for feeding to ruminant livestock in the USA.

### *Ruminal protein degradation*

Comparison of ruminal protein degradation data (Table 2) between standard hay and standard hay + TCA indicated no differences in any parameter estimated. It was assumed that, if there were no direct effects of TCA addition on microbial protein degradation, then none of the acids directly affected microbial protein degradation. Therefore, all acid treatments will be discussed in terms of their effects on forage proteins rather than on ruminal microbial metabolism.

The crude protein in fraction B, as estimated by these procedures, comprised all N that was not present initially in $NH_3$ or free AA (i.e. fraction B included peptide-bound AA N). Fraction B in acid-treated hays (mean = 926 g N kg$^{-1}$ total N) was greater ($P < 0{\cdot}05$) than that in fresh and control hay (mean = 918 g N kg$^{-1}$ total N). However, the difference of only 1% between the two hays suggested that the quantity of intact plant protein in the standing forage (represented by fresh forage) was essentially unchanged by hay-making in this trial. Fraction B in all silages was lower ($P < 0{\cdot}05$) than that in fresh forage, reflecting the NPN formation during ensiling. However, the proportion of fraction B in TCA silage (902 g N kg$^{-1}$ total N) was 98% of that in fresh forage and indicated that TCA gave nearly complete preservation of intact plant proteins. Fraction B was similar between formic and sulphuric acid silages (mean = 754 g N kg$^{-1}$ total N), and represented a 60% increase over the control silage (472 g N kg$^{-1}$ total N).

Rates of ruminal protein degradation ($k_d$) obtained with the LS methodology were highest ($P < 0{\cdot}05$) and equal for the fresh forage and control hay (mean = 0·114 h$^{-1}$), indicating that there was no effect on protein degradability of normal hay-making procedures. Degradation rates of all acid-treated forages were not different ($P > 0{\cdot}05$) from one another (mean = 0·084 h$^{-1}$). The similarity of degradation rates among acid treated forages, preserved either as hay or silage, indicated a consistent effect of acid rendering native plant proteins less susceptible to ruminal microbial attack. Moreover, the fact that TCA-treated forage had degradation rates similar to those of forages treated with sulphuric or formic acids suggested that its effect was mediated not by protein precipitation, but rather by

**Table 2** *In vitro* ruminal protein degradability of lucerne forages†

| | Fraction B | $k_d$ (h$^{-1}$) | | Estimated escape (g kg$^{-1}$ total N) | |
|---|---|---|---|---|---|
| Forage | (g kg$^{-1}$ total N) | LS | IMM | LS | IMM |
| Standard hay‡ | 908 | 0·079 | 0·116 | 391 | 310 |
| Standard hay + TCA‡ | 910 | 0·078 | 0·121 | 397 | 303 |
| Fresh | 917$^{b}$ | 0·120$^{a}$ | 0·172$^{a}$ | 307$^{d}$ | 240$^{c}$ |
| Silages | | | | | |
| Control | 472$^{f}$ | 0·020$^{c}$ | ND§ | 359$^{bc}$ | ND§ |
| Sulphuric acid | 757$^{d}$ | 0·086$^{b}$ | 0·140$^{bc}$ | 312$^{d}$ | 228$^{c}$ |
| Formic acid | 750$^{e}$ | 0·088$^{b}$ | 0·136$^{bc}$ | 307$^{d}$ | 232$^{c}$ |
| TCA | 902$^{c}$ | 0·087$^{b}$ | 0·126$^{bc}$ | 368$^{b}$ | 291$^{ab}$ |
| Hays | | | | | |
| Control | 919$^{b}$ | 0·108$^{a}$ | 0·144$^{b}$ | 330$^{cd}$ | 274$^{b}$ |
| Sulphuric acid | 926$^{a}$ | 0·086$^{b}$ | 0·121$^{bc}$ | 381$^{ab}$ | 308$^{a}$ |
| Formic acid | 926$^{a}$ | 0·082$^{b}$ | 0·116$^{c}$ | 392$^{ab}$ | 316$^{a}$ |
| TCA | 928$^{a}$ | 0·077$^{b}$ | 0·121$^{bc}$ | 406$^{a}$ | 312$^{a}$ |
| s.e.m. | 2 | 0·005 | 0·008 | 11 | 11 |

Means within columns not sharing a common letter differ ($P < 0{\cdot}05$).

†LS, limited substrate method; IMM, integrated Michaelis–Menten method; TCA, trichloroacetic acid.

‡These substrates were evaluated separately from the remainder of the data (see text). No differences ($P > 0{\cdot}32$) were found in any parameters estimated.

§Not determined because IMM estimates were unstable (see text).

some common action of acidification. Lastly, consistent with previous observations using the inhibitor *in vitro* method (Broderick, 1984), the ruminal degradation rate for fraction B protein in control lucerne silage (0·020 $h^{-1}$) was similar to that found with certain resistant proteins, such as expeller soybean meal. Fraction B in the control lucerne silage consisted of proteins that survived proteolysis during ensiling; these proteins would be expected to be more resistant to ruminal proteolytic attack. Thus, the higher $k_d$ values obtained with the acid-treated forages, fresh forage and control hay probably resulted from the degradation of the same, relatively labile, native plant proteins that were no longer present in the B fraction of control silage, whereas the $k_d$ for control silage derived from degradation of the more resisant plant proteins.

Estimates of protein escaping the rumen undegraded are a function of both the quantity of fraction B and its degradation rate. Relative to fresh forage, the estimates of ruminal undegraded protein were greater ($P < 0{\cdot}05$) in TCA and control silages, and not different ($P > 0{\cdot}05$) for sulphuric and formic acid silages (Table 2). The greater amount of ruminal undegraded protein in the control silage was due to its very low degradation rate, the $k_d$ being only one-sixth that of fresh forage. This substantial decrease in $k_d$ was sufficient to increase the estimate of ruminal protein escape for the control silage, despite its extensive breakdown during ensiling of native plant proteins and its relatively small fraction B. This anomalous result was probably related to the high background levels of free AAs and peptides in the NPN fraction of the control silage; end product inhibition due to high AA and peptide concentrations may have suppressed the measured degradation rates. Further, the inhibitors used in the LS incubations prevented microbial uptake of $NH_3$, free AAs and peptides (Broderick, 1987); these inhibitors would also prevent catabolism of the high background levels of free AAs and peptides in the control silage. Thus, underestimation of degradation rate due to end product inhibition may be greater in the control silage than in the formic acid- and sulphuric acid-treated silages. Although both formic and sulphuric acids reduced degradation rates relative to fresh forage, the smaller fraction B content of these forages resulted in no net change in estimated protein escape. Relative to fresh forage, acid treatment of hay decreased ($P < 0{\cdot}05$) degradation rate and increased ($P < 0{\cdot}05$) estimated protein escape. However, neither degradation rate nor estimated protein escape of the control hay differed ($P > 0{\cdot}05$) from that of the fresh forage (Table 2). The increased content of escape protein in acid-treated forages clearly was mediated by the lower degradation rate in these forages (mean $k_d$ was 68% of fresh forage). Similar content of escape protein between control hay and fresh forage reflected the similar values for fraction B and $k_d$ in these forages.

Consistent with previous observations (Broderick and Clayton, 1992), degradation rates estimated by IMM were greater than those estimated by the LS methodology. This may have resulted partly because the IMM method allowed for estimation of $k_d$, defined as the tangent to the curve of velocity vs. substrate concentration [S], in accordance with the first-order assumption ([S] « $K_m$). However, degradation rates for control silage were unstable with IMM methodology and the model failed to converge in two of three instances for that forage. In both the IMM and LS methods, release of additional $NH_3$ and free AAs was measured against background concentrations of these metabolites. Because the IMM method required adding increasing amounts of substrate, large errors in estimating extent of degradation due to the high backgrounds of free AAs in control silage may have prevented convergence of the IMM model. Despite quantitative differences in degradation rate and undegraded protein estimated by the LS and IMM procedures, the pattern of treatment effects observed using the two methods was similar.

Sulphuric and formic acids had similar effects on NPN formation during ensiling and on the extent of ruminal protein degradation. Trichloroacetic acid was more effective than sulphuric or formic acids in reducing NPN formation, probably through inhibiting plant proteases (White *et al.*, 1968) during ensiling, thereby increasing the amounts of fraction B and ruminal undegraded protein. However, the safety of treating silage with TCA has not been established. Evaluation of the effects of acid treatment on ruminal degradation of silage protein was confounded by the difficulty of obtaining reliable estimates of rate and escape for the control silage. Nevertheless, results from this experiment indicated a clear effect of acid treatment on reducing breakdown of native plant proteins during ensiling and in reducing the rate of ruminal protein degradation relative to fresh forage. Rates of ruminal protein degradation in acid-treated hay were only 76% or 83% of control hay, as estimated by LS or IMM methodology respectively; all acid treatments yielded similar reductions in degradation rate.

## Conclusions

Treatment of lucerne with sulphuric and formic acids reduced proteolysis to similar extents during both ensiling and *in vitro* ruminal incubations; neither acid was as effective as TCA in reducing proteolysis in the silo. Thus, it appeared that the previously observed effects of formic acid on improving utilization of protein in wilted lucerne silage by ruminant livestock (Nagel and Broderick, 1992) were not due to destruction of plant proteases, but were due solely to acidifying the forage below the pH optima of plant proteases. It must be noted that the safety of treating silage with TCA has not been established.

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